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[7] Random Cloning and Sequencing by the M13/Dideoxynucleotide Chain Termination Method

By A. T. BANKIER, K. M. WESTON, and B. G. BARRELL

Introduction

The dideoxy chain terminator/M13 vector method of DNA sequencing[1,2] is widely considered to be the quickest method of determining the sequence of large fragments of DNA. Lengths of DNA are cloned into the bacteriophage M13, which provides a source of large quantities of single-stranded DNA. This single-stranded DNA can then be used as a template in a primer extension dideoxynucleotide sequence reaction.[3]

The maximum length of sequence which can be read using currently available separation methods is around 500 bases. This means that to determine the sequence of fragments greater than this length, separate specific priming reactions along the insert or separate cloning experiments are necessary. Synthetic oligonucleotide primers which are complementary to the template DNA at the limit of the determined sequence can be used to further extend the known sequence. However, this approach is both costly and time consuming, as primers need to be synthesized and purified for each sequence reaction. An alternative is to engineer progressive deletions of the insert to bring regions of increasing distance from one end, adjacent to the universal primer site (see other articles, this volume). The major drawback of both of these strategies is the requirement that the entire DNA fragment be cloned into M13, which proves to be an unstable vector for inserts much greater than 2 kb, and even for some smaller specific sequences. Additionally, because of common secondary structure problems in sequencing, the whole strategy has to be performed on the template in both orientations. Many of the described ordered deletion methods suffer from other problems, such as a requirement for recloning since deletions occur from both ends, or a dependence upon specific restriction endonuclease sites.

It is possible to overcome all of these difficulties by breaking down the fragment to be sequenced into subfragments which can be easily cloned

[1] F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
[2] J. Messing, B. Gronenborn, B. Muller-Hill, and P. H. Hofschneider, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3642 (1977).
[3] F. Sanger, A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe, *J. Mol. Biol.* **143**, 161 (1980).

into M13. The sequences obtained can then be combined and overlapped until the entire fragment is sequenced.

The simplest method of breaking a DNA molecule into subfragments is by using restriction endonucleases. However, this suffers from the following problems:

1. The distribution of sites is uneven, as some areas may be totally devoid of sites. This produces a wide range of subfragment sizes from the very small to the very large.
2. Because it takes the same effort to sequence a 10-base insert as it does a 300-base insert, small fragments are best avoided.
3. The small fragments clone much more readily into M13, and since they arise from very specific regions, the same region tends to be covered many times.
4. In order to obtain overlaps between subfragments, several libraries, each using a different enzyme, need to be prepared.

Unless a detailed and accurate restriction map is available for the original fragment, or if it is very small (around 1 kb), it is better to use a more random method to generate the subfragments. Random fragments can be made by using less sequence-specific enzymes such as DNase I,[4] or by mechanical means such as sonication.[5] Sonication would normally be chosen, since once a particular machine is calibrated, it can be used for any size fragment in any concentration, whereas using enzymes requires careful calibration for each experiment.

We describe here a series of protocols to enable the sequencing of fragments within the range of 1 to 20 kb, using this sonication, or shotgun, procedure. Many of the techniques can of course be applied to other dideoxy/M13 strategies. In particular, using a microtiter plate as the reaction vessel can dramatically increase the rate at which the sequence can be determined, and has opened up the possibility of automation using commercially available equipment.

The sonication method, due to its random nature, results in the sequence of each base in a DNA fragment being determined on average six to eight times before a project is completed. This redundancy, a built-in safeguard against sequencing errors, is compensated for by the speed of the sequencing technique; it is relatively painless to sequence 40–60 templates daily, and so the rate of accumulation of sequence data remains rapid. A flow chart of the whole procedure is shown in Fig. 1.

[4] S. Anderson, *Nucleic Acids Res.* **9**, 3015 (1981).
[5] P. L. Deininger, *Anal. Biochem.* **129**, 216 (1983).

FIG. 1
step are

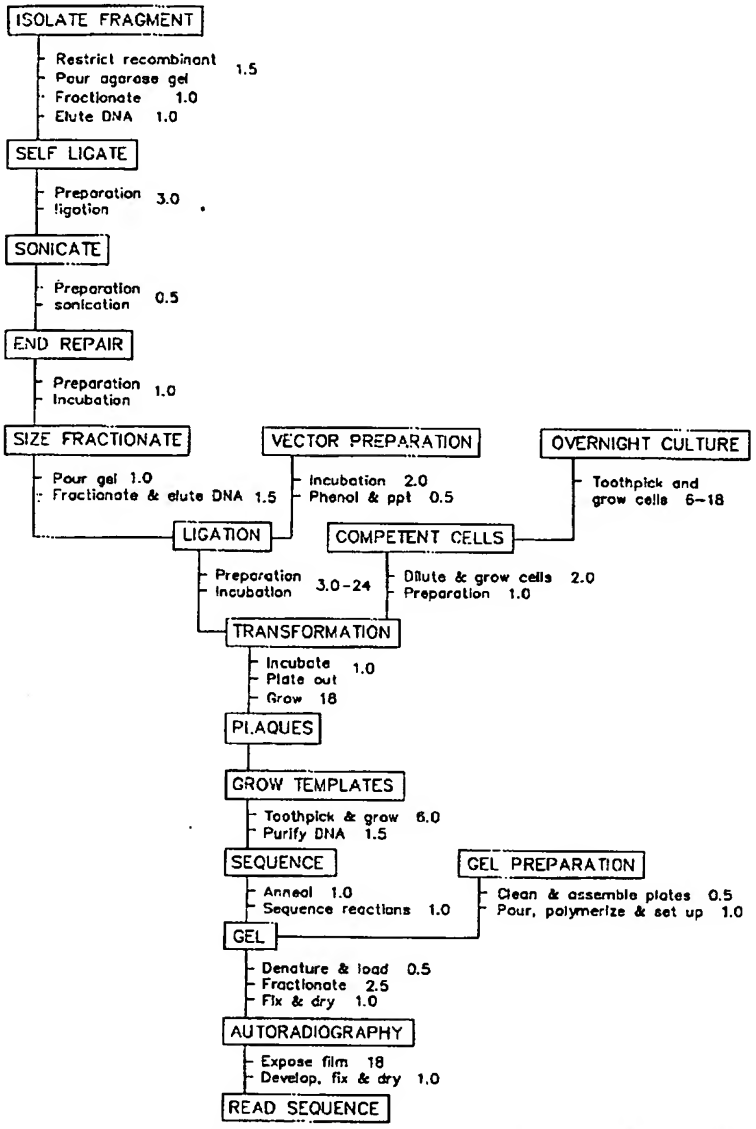

FIG. 1. A flow chart for shotgun cloning and sequencing. The approximate times for each step are given in hours.

Randomly Generated Subfragments

At the start of the sequencing project, the DNA to be analyzed must be isolated and then sheared to produce a random set of subfragments covering the entire region of interest. A miniprep using one of the widely accepted protocols[6] will normally yield sufficient DNA from which to purify the fragment to be sequenced. If, however, the fragment is small compared with the vector, a larger quantity may have to be isolated. Several thousand subclones can be generated from 2–5 μg of DNA, which is therefore enough for even the largest of sequencing projects. As the loss of a significant proportion of the starting material is unavoidable during the generation of subfragments, it is inadvisable to use less than 2 μg of DNA.

The easiest method of purifying the fragment to be sequenced from the cloning vector is cleavage with suitable restriction endonuclease(s) followed by fractionation of the restricted DNA and isolation from a low-gelling-temperature agarose minigel. A gel size of 10 × 10 cm is convenient, but even smaller formats can be used without difficulty. The gel is run in the presence of ethidium bromide and the fragment band is cut out as soon as it has separated cleanly from the vector band. The DNA is liberated from the agarose by melting and phenol extraction. An alternative approach to isolating the fragment is random subcloning of the entire recombinant. The clones produced can then be screened by hybridization to identify those containing fragment sequences. All those having only vector sequences are disregarded. This screening procedure increases the time and effort needed to complete a sequence and would not normally be considered as an alternative unless the vector and the insert restriction fragments are of too similar a size to be separated by gel electrophoresis.

A random set of subfragments is generated by shearing the purified DNA in a normal laboratory sonicator. Mechanical shearing by sound results from the two ends of a fragment of DNA being moved in opposing directions by sound waves set up in the solvent. The maximum shear energy is experienced around the center of the molecule. If a fragment is sheared in this way, subfragments arise by an approximate halving of the original and of each subsequent shear product. To increase the random nature of the process, the DNA should be self-ligated before sonication.

The products of self-ligation can be either circles or chains formed of one or several units' length. In the case of circles, there are no ends and so the position of the first break is totally random, and the ends of the subfragments produced will have a totally random distribution. When

[6] H. C. Birnboim and J. Doly, *Nucleic Acids Res.* **7**, 1513 (1979).

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concatenated chains are formed by self-ligation, the randomness is increased by virtue of an increase in the region covered by the distribution of break points around the center, the area covered becoming larger with longer chains.

Although any sonicator can be used, those having a cup horn probe avoid the risk of sample cross-contamination, as the DNA can be sheared in a microfuge tube in isolation from the probe. If the probe is in direct contact with the sample solution, it must be carefully cleaned both before and after use by boiling in detergent.

As the products of shearing become smaller, the effective shear forces decrease and there is resistance to further breakage. The effect of this is a rapid drop in fragment size followed by a plateau where the size drops comparatively slowly. Both the time taken to reach the size plateau and the average length of DNA fragments produced at this point will depend largely on the total power output of the sonicator. The time taken to reach the plateau is, however, reasonably independent of the length of the original fragment. So, once calibrated to produce a suitable range of fragment sizes, a sonicator can be used over a wide range of DNA lengths, concentrations, and solution volumes reproducibly.

A simple time course of sonication, at maximum output, using any available DNA as the test sample, will serve to calibrate a particular sonicator. If small aliquots of each time point are fractionated side by side on an agarose gel, the most suitable time can be easily determined. When using a cup horn probe sonicator of power output around 375 W, a suitable time will be close to 160 sec. Direct contact probes will need less than 25 sec.

Procedure 1: Isolation of Fragment

Recipes

Restriction enzyme buffer: as recommended by supplier

10× TBE:
108 g Tris base
55 g boric acid
9.3 g EDTA
Make up to 1 liter with deionized water.

1.0% HGT agarose gel mix:
25 ml 1.0× TBE
0.25 g HGT agarose
Dissolve at 100° and add 1 μl of 10 mg/ml ethidium bromide just before pouring.

0.8% LGT agarose gel mix:
50 ml 1.0× TBE
0.4 g LGT agarose (B.R.L. ultrapure low-melting-point agarose)
Dissolve at 100° and add 2 μl of 10 mg/ml ethidium bromide just before pouring.

TBE dye mix (100 ml):
10 ml 10× TBE
0.1 g bromphenol blue
20 g sucrose
Make up to 100 ml with water.

TE buffer:
10 m*M* Tris (pH 8.0–8.5)
0.1 m*M* EDTA (Na_2)

1. Take sufficient recombinant DNA to yield ~5 μg of target DNA, i.e.,

$$\text{(length of vector + target)/length of target} \times 5 \quad \mu g$$

2. Cleave the recombinant with a suitable restriction enzyme(s) to excise the target DNA. Use the suppliers' recommended conditions with a twofold increase in the recommended enzyme concentration and digest for 1.5–2 hr. (The final volume to be around 100 μl.)
3. Into a 100-ml conical flask put 0.25 g of HGT agarose and 25 ml of 1× TBE, and dissolve by placing in a microwave oven or in a boiling waterbath with stirring (~10 min).
4. Remove the flask from the heat and mix in 1 μl of 10 mg/ml ethidium bromide.
5. Pour the gel in a 10 × 10-cm minigel apparatus, put in the slot former (slot width ~0.3 cm), and leave the gel to set for around an hour at 4° before using.
6. Pour on 25 ml of 1.0× TBE + 1 μl of 10 mg/ml ethidium bromide and slowly remove the slot former.
7. To assay the digest, add 1/10 volume of TBE dye to ~0.2 μg of digested DNA and load onto the minigel. Also load a suitable size marker.
8. Run at 50 mA until the degree of digestion can be assessed.
9. Pour a 50-ml 0.8% LGT agarose minigel using 2-cm-wide slot formers, and allow the gel to set for an hour at 4° (modify steps 3–6 above).
10. When the digestion is complete, load the DNA onto the LGT agarose minigel, using no more than 10 μg per slot.
11. Connect the minigel to a power supply and run at 30 mA. Monitor the running of the gel under UV light periodically until the required band has separated cleanly from the other band(s).

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12. Cut out the band with a safety razor blade or scalpel, put the gel slice into a 1.5-ml microfuge tube, and melt in a 70° water bath or heat block.
13. Add an equal volume of TE-saturated phenol, vortex well, and leave for 5 min. Vortex again and centrifuge for 2 min in a microfuge.
14. Remove the upper aqueous layer and re-extract the phenol phase with an equal volume of TE.
15. Combine the aqueous phases and phenol extract a further two or three times or until a clear interface is obtained.
16. Add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of ethanol, and precipitate in dry ice/isopropanol for 20 min.
17. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.
18. Add 1 ml of 95% ethanol, centrifuge for 5 min, and pour off the ethanol.
19. Vacuum dry the pellet and redissolve in 20 μl of TE.

Specific batches of some low-gelling-temperature agaroses occasionally affect the isolated DNA such that it cannot be ligated or cleaved with restriction enzymes. In extreme cases, samples of the DNA will not enter agarose gels during electrophoresis. It is best to use ultrapure agarose and to minimize the amount of agarose taken as a gel slice to try to decrease on the carryover of the contaminant. Run the agarose gel only for a time sufficient to separate the band of interest, and when cutting out the band, trim off the excess agarose.

Procedure 2: Fragment Self-Ligation

Recipes

10× ligase buffer:
500 m*M* Tris (pH 7.5)
100 m*M* $MgCl_2$
100 m*M* DTT

1. To the isolated fragment in 20 μl of TE add
3 μl 10× ligase buffer
3 μl 10 m*M* rATP
2 U T4 DNA ligase (New England Biolabs)
Bring volume to 30 μl with water.
2. Incubate at 15° for 2–3 hr.

If the DNA to be sequenced was isolated using two restriction endonucleases which produce different cohesive ends, self-ligation will produce

chains of fragments in alternating orientation. The junctions are therefore the centers of large, inverted repeats. Such potential structures are rarely stably cloned in M13 vectors and the fragment ends will rarely be represented in the final sequence. A simple solution to this problem is to exclude around 10% of the starting material from the self-ligation and then to recombine the two parts before sonication.

Procedure 3: Sonication

Using a Heat Systems Ultrasonics W-375 Cup Horn Sonicator

1. Fill the cup horn of the sonicator with water to a depth of ~3 cm.
2. Clamp the Sarstedt tube containing the self-ligated fragment in 30 μl ~1 mm above the probe.
3. Set at maximum output and sonicate for a total of 2 × 80 sec. After each 80 sec, centrifuge the tube briefly to concentrate the DNA solution and change the water in the probe to help keep it cool.
4. Between bursts, turn down the output to minimum and keep the sonicator switched on to keep the cooling fan working.
5. Run a 1- to 2-μl aliquot on a minigel to check the extent of sonication. Use *Sau*3A-cut pBR322 as a marker and aim for a peak size distribution of around 300–1000 base pairs.

Using Any Other Sonicator

1. Precalibrate the sonicator using any available DNA (e.g., M13 RF) to give the required size range (as a first attempt try a total of ~20 sec). Use the minimum volume which can be sonicated with the smallest available probe.
2. Dismantle the probe and boil it in 1% SDS to clean it and to remove any contaminating DNA.
3. Wash the probe thoroughly with deionized water to completely remove the SDS.
4. Dilute the self-ligated fragment with TE to the minimum volume which can be sonicated.
5. Sonicate the fragment on ice, in short bursts (~1/4 of total duration), for the appropriate total time. Between each burst allow the sample to cool for 2–3 min to avoid overheating.
6. Add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of ethanol, and precipitate in dry ice/isopropanol for 20 min.
7. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.
8. Add 1 ml of 95% ethanol, centrifuge for 5 min, and pour off the ethanol.
9. Vacuum dry the pellet and redissolve in 30 μl of TE.

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Sarstedt microfuge tubes have thinner walls than some other 1.5-ml tubes. If using a different variety of tube, required sonication times may be longer.

When sonicating, do not allow the DNA solution to warm up, as the sonication characteristics of DNA change at higher temperatures.

If a cup horn probe has been used, it is not necessary to phenol extract or ethanol precipitate the DNA after sonication. The volumes and buffers recommended here enable the sample to be processed with a minimum of unnecessary manipulations.

Subfragment End Repair

The molecule ends resulting from sonication, as might be expected, are not of a form which can be ligated directly into the subcloning vector. Before cloning the subfragments into any vector, their ends need to be repaired. The most common approach is to use enzymes to produce blunt ends which can be directly ligated to a suitable flush-ended vector. Alternatively, end-repaired subfragments can be ligated to restriction endonuclease linkers, cleaved with restriction enzyme, and cloned into an appropriately cut vector.

Several different enzymes have been tried in an attempt to maximize the efficiency of end repair: mung bean, S_1, and *Bal*31 nucleases, and DNA polymerases such as T4 or Klenow. All of these enzymes, even when used in combination, result in comparable efficiencies. At best, this is less than 1% of the cloning efficiency of fragments generated by using restriction endonucleases. These very low yields are presumably due to the enzymes' inability to deal with severely disrupted fragment ends or perhaps the internal damage produced by the process of sonication, which cannot be repaired by the host upon transformation.

In practice it is probably easier to use polymerases, as they do not require careful calibration, and, since the subclones are to be used in DNA sequencing, a supply of good-quality Klenow fragment DNA polymerase should be at hand. T4 DNA polymerase has a greater 3′ exonuclease activity than the Klenow polymerase and when the two enzymes are used in conjunction, good results can be obtained using short incubation times at room temperature.

Procedure 4: Fragment End Repair

Recipes

dNTP chase:
0.5 m*M* dTTP
0.5 m*M* dCTP

0.5 m*M* dGTP
0.5 m*M* dATP
From 50 m*M* stocks in TE

1. To the self-ligated/sonicated fragment in 30 μl add
   2 μl 0.5 m*M* dNTPs (sequence chase)
   10 U Klenow DNA *Pol*I (Boehringer Mannheim)
   10 U T4 DNA *Pol*
2. Incubate at room temperature for 30 min.

Whatever the reasons for poor efficiencies, the end-repair step is likely to be the reason for any total failure in random cloning by sonication. If enzymatic repair is inhibited, the yields of recombinants can be insignificant. As a first attempt at resolving a complete failure, try performing the end-repair step again. In this case, $MgCl_2$ to a final concentration of around 5 m*M* should be added to the above recipe, as both enzymes involved require Mg^{2+} as a cofactor.

Size Selection

One of the advantages of random cloning is the ability to control the size of the subfragment produced by altering sonication parameters. The size range of the fragment chosen for cloning, however, needs further control.

Two factors determine the minimum size to be selected. First, the insert to be sequenced should be at least as long as the maximum which can be read from a normal sequence gel run. If this is not the case, much time and effort will be wasted in producing useless vector sequences. Second, since the subfragments have ends generated at random, and not at specific primary sequence sites, as with restriction enzymes, it is not possible to detect the junctions of religated noncontiguous fragments simply by inspection. If there is any chance of cloning multiple inserts, the junctions need to be outside the range normally sequenced. The second point is not too critical, as the same redundancy of sequence which makes random cloning/sequencing so reliable, in terms of the quality of data, also ensures that such noncontiguous junctions will be obvious in the accumulating sequence, although it may cause interpretation problems in the early stages.

There are also constraints on the upper size range selected for cloning. Large inserts can be difficult to clone in M13 vectors, and even when cloned, they can be unstable, giving rise to deletions. Also, it is often useful to know the total region covered by a particular insert. For example, toward the end of a project, when trying to determine the entire

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sequence on both strands, it possible to cover gaps, by using the reverse sequence primer, or to reverse the orientation of an insert to get sequence information from the other strand. The M13 clone library produced during the sequencing can be a very useful source of probes for hybridization or S_1 protection. For these methods, knowing the insert length and area of sequence covered can be very important.

In practice, a convenient size range for subcloning is 300–600 bp. This ensures a maximum of information from each sequence gel run, but still allows identification of the end of the insert.

The size selection can be done easily using a low-gelling-temperature agarose minigel. If the repaired fragments are fractionated alongside DNA size markers such as *Sau*3A-cut pBR322 or pAT153, a gel slice containing a good size range can be removed with a scalpel. The gel slice is then melted at 70° and the DNA is phenol extracted and ethanol precipitated. The whole process takes 1–2 hr and gives a reasonably high yield of sufficiently pure fragments for cloning.

There are several alternatives to using low-gelling-temperature agarose as a means of recovering fractionated DNA from agarose gels. A slot can be cut at the lower size limit and a series of short electrophoresis bursts followed by buffer changes in the slot will electroelute the DNA. Or a piece of DEAE paper placed within the slot will collect the DNA, which can subsequently be eluted. A gel slice can also be removed, put in dialysis tubing, and submerged, within an electric field; the DNA will be electroeluted out of the gel slice and can be recovered from the buffer surrounding the slice within the dialysis bag.

Procedure 5: Size Fractionation

Recipes

1.5% HGT gel mix:
50 ml 1.0× TBE
0.75 g HGT agarose
Dissolve at 100° and add 2 μl of 10 mg/ml ethidium bromide just before pouring.

0.8% LGT gel mix: as in procedure 1.

Using LGT Agarose

1. To the end-repaired fragments add 5 μl of TBE dye mix and load onto a 50-ml 0.8% low-gelling-temperature agarose/TBE minigel, using a 1-cm-wide slot former.
2. Connect to a power supply and run at 30 mA until the bromphenol blue marker dye has entered ~1 cm into the gel.

3. Cut out the required size ranges (300–600 and 600–1000 bp) with a safety razor blade or scalpel, put the gel slices into separate 1.5-ml microfuge tubes, and melt in a 70° water bath or heat block.

4. Add an equal volume of TE-saturated phenol, vortex well, and leave for 5 min. Vortex and centrifuge 2 min in a microfuge.

5. Remove upper aqueous layer and re-extract the phenol phase with an equal volume of TE.

6. Combine the aqueous phases, keeping each size fraction separate, and phenol extract both fractions a further two or three times or until a clear interface is obtained.

7. Add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of ethanol, and precipitate in dry ice/isopropanol for 20 min.

8. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.

9. Add 1 ml of 95% ethanol, centrifuge for 5 min, and pour off the ethanol.

10. Vacuum dry the pellets and redissolve each in 50 μl of TE.

Using HGT Agarose

1. To the end-repaired fragments add 5 μl of TBE dye mix and load onto a 50-ml, 1.5% high-gelling-temperature agarose/TBE minigel, using a 1-cm-wide slot former. Use only sufficient running buffer to make an electrical connection, trying to keep the top of the gel dry.

2. Connect to a power supply and run at 30 mA until the bromphenol blue marker dye has entered 1–2 cm into the gel.

3. Switch off the power and with scalpel cut a 1-mm slot in front of the lower limit of the size range required (250–300 bp), using *Sau*3A-cut pBR322 as a marker.

4. Fill the collection slot with TBE and connect the minigel to the power supply.

5. Run at 20 mA for 45 sec, switch off the power, and remove the TBE from the slot.

6. Repeat steps 4 and 5, combining the fractions until the upper size of the required range is removed (600–1000 bp).

7. Add an equal volume of TE-saturated phenol, vortex, and leave for 5 min. Vortex again and centrifuge 2 min in a microfuge.

8. Remove upper aqueous layer and add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of 95% ethanol, and precipitate in dry ice/isopropanol for 20 min.

9. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.

10. Add 1 ml 95% ethanol, centrifuge 5 min, and pour off the ethanol.

11. Vacuum dry the pellet and redissolve in 50–100 μl of TE.

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As at other times when using low-gelling-temperature agarose to recover samples, use only commercially available ultrapure varieties and minimize the amount of extra agarose carried over.

If end repair is attempted after size fractionation, the overall size range will be reduced. This probably results from the removal of single-stranded ends.

Using the trough elution technique can be very tedious, as it requires many changes of buffer, especially if the sample has migrated any further than 1–2 cm into the gel. The overall number of fractions collected can be reduced by replacing the buffer in the slot with 5× TBE. The effect of this is rather like a buffer gradient gel. The voltage drop across the high buffer-containing slot is less than in the rest of the gel, and so DNA molecules entering this high buffer zone slow down and take longer to migrate across the slot. A snag with this technique is that if many slot buffer changes are needed, the high buffer front migrates into the gel above the slot and the DNA is slowed down even before it enters the slot.

Cloning into M13

M13 is the vector of choice for didcoxy sequencing for two main reasons. First, M13 bacteriophages are packaged single strands of DNA which are extruded from infected *Escherichia coli* cells into the surrounding culture medium. This means that considerable quantities of single-stranded template DNA can be easily produced. Second, the M13 mp vectors have a quick color assay to identify bacterial cells infected with phage containing an insert.

The M13 mp series of vectors was constructed by insertion of a restriction fragment of the *E. coli lac* regulatory region into wild-type M13. This fragment contains the region coding for the first 145 amino acids of the α-peptide of the β-galactosidase gene. Within this region synthetic oligonucleotides, containing several unique restriction enzyme sites, have been introduced such that the α-peptide reading frame is retained. When these phages infect defective *E. coli* (F′, Δ*lac pro*), which have a deletion within this region of the β-galactosidase gene, complementation occurs and a functional β-galactosidase is produced. In the presence of IPTG, the substrate Xgal is hydrolyzed by β-galactosidase to bromochloroindole, which confers a blue color to the infected plaque on a bacterial lawn. If, however, an insert is cloned into one of the synthetic oligonucleotide restriction sites such as to interrupt the α-peptide coding region, no functional β-galactosidase is produced, the Xgal is not hydrolyzed, and the infected plaque remains colorless. This makes detection of recombinants very simple.

The M13mp vectors provide several unique restriction sites for clon-

ing. The decision of which vector to use will largely depend upon whether it has the required restriction sites. If the vector is to be used primarily for sequencing, the position of the cloning site relative to the "universal" primer site is important. If it is too close, obtaining sequence information at the start of an insert can be difficult. If it is too distant, sequence gels will have to be run considerably longer before the beginning of the insert is at the bottom of the gel. For reasons of biohazard containment, it may also be necessary to choose a vector with amber mutations which require a suppressor host. The blunt-ended fragments from the sonication/end repair/size fractionation can be cloned directly into M13 restricted with a suitable blunt-cutting restriction endonuclease such as *Sma*I-cut M13mp8. This restricted vector can be prepared in bulk and stored as aliquots for long periods at −20°. Phosphatasing the linearized vector prevents recircularization without an insert, dramatically reducing the background of nonrecombinant M13-infected plaques. The phosphatase step can most conveniently be carried out concurrently with the restriction enzyme cleavage.

The most reliable assay of the quality of prepared vector is in transformation. Since all of the reagents can be stored for long periods at −20°, each cloning experiment can be carefully controlled. Ideally, standard solutions of buffers, vector, and fragments should result in reproducible numbers of blue/colorless plaques.

The following three controls should be carried out with each ligation/transformation experiment. First, transformation with the unligated prepared vector indicates the extent of restriction; the fewer the number of blue plaques, the more complete is the cleavage. Second, if the same quantity of vector is self-ligated, the increase in blues reflects incomplete phosphatasing. Any colorless plaques in this vector self-ligation control arise from vector with deletions within the β-galactosidase coding region, usually a single nucleotide at the restriction enzyme site, causing a frameshift. Last, a ligation of vector with a standard solution of fragments (*Alu*I-cut λ DNA ligates very efficiently) will not only assess the quality of the vector, but provides a good test of how the transformation step has gone, as the numbers of plaques obtained should not vary too much between cloning experiments. Variations in the total numbers arise from the variable quality of the competent cells.

Even using standard solutions and protocols, the number of plaques can vary wildly from one day to the next, the most variable aspect being the competent cells. Vigorously growing mid-log-phase cells are essential for good results. Stock cultures should be restreaked regularly. During their preparation, competent cells need to be treated gently and, once prepared, are best used immediately. The poor yields associated with

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sonicated fragments in cloning makes the use of high-efficiency transformation procedures very important. An increase in transformants of around two orders of magnitude has been reported using the protocol of Hanahan.[7] Certainly an increase of 10-fold is routine.

The number of recombinant plaques obtained in cloning experiments with randomly generated subfragments will be much lower than those of the restriction fragment control. A much higher fragment concentration has to be used in order to get enough clones for sequencing, but there is a limit to how high this concentration can be taken. The number of colorless plaques will increase with increasing fragment concentration until a peak is reached beyond which the numbers will drop as concatenation exceeds circularization. Using a range of fragment concentrations gives us some idea of the amount needed to yield a maximum of clones.

Procedure 6: Vector Preparation

Recipes

10× *Sma*I buffer:
150 m*M* Tris (pH 8.0)
50 m*M* $MgCl_2$
150 m*M* KCl

Procedure

1. To a 1.5-ml microfuge tube add
5 μl M13mp RF DNA (1 μg/μl)
5 μl 10× *Sma*I buffer
20 U calf alkaline phosphatase (Boehringer-Mannheim)
40 μl water
5 U *Sma*I (15-min units)
Incubate 1 hr at 37°.
2. Assay cleavage on 1.0% HGT agarose minigel.
3. Add an equal volume of TE-saturated phenol, vortex well, and leave for 5 min. Vortex and centrifuge 2 min in a microfuge.
4. Remove upper aqueous layer and add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of ethanol, and precipitate in dry ice/isopropanol for 20 min.
5. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.
6. Add 1 ml of 95% ethanol, centrifuge for 5 min, and pour off the ethanol.

[7] D. Hanahan, *J. Mol. Biol.* **166**, 557 (1983).

7. Vacuum dry the pellet and redissolve in TE to 20 ng/μl.
8. Store in 50-μl aliquots at −20°.

Damage to vector ends, giving rise to "false" colorless plaques, increases with overdigestion with some lots of restriction enzymes. The low costs involved make it advisable to simply repeat a vector preparation if too many "false" plaques are seen, rather than wasting time growing and sequencing them.

The conditions used are not ideal for calf alkaline phosphatase but are usually satisfactory. If problems are encountered, restrict with *Sma*I, ethanol precipitate, and then phosphatase using the suppliers' recommended conditions. Some preparations of calf alkaline phosphatase are contaminated with exonuclease activity and will produce vector with nibbled ends. Again, phosphatasing separately in the absence of Mg^{2+} overcomes this problem.

Procedure 7: Ligation

Recipes

10× ligase buffer:
500 m*M* Tris (pH 7.5)
100 m*M* $MgCl_2$
100 m*M* DTT

1. In a 1.5-ml microfuge tube make a vector/buffer mixture by adding
35 μl water
6 μl 10× ligase buffer
6 μl 10 m*M* rATP
6 μl *Sma*I-cut M13mp vector (120 ng)
2. To 1.5 ml microfuge tubes add the following components:

	Tube number					
	1	2	3	4	5	6
Vector/buffer mix (μl)	8	8	8	8	8	8
End-repaired fragments (μl)	1	2	3	—	—	—
*Alu*I λ fragments (μl) (10 ng/μl)	—	—	—	1	—	—
T4 DNA ligase (U) (New England Biolabs)	100	100	100	100	100	—

3. Incubate at 15° overnight.

: in TE to 20 ng/μl.

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lowing components:

	Tube number				
	2	3	4	5	6
	8	8	8	8	8
	2	3	—	—	—
	—	—	1	—	—
	100	100	100	100	—

Shorter ligation periods at 15 or 20° are possible with resulting reduced yields. Performing the ligation at room temperature for around 3 hr will usually give around 20% fewer recombinants. The advantages of time saved will often justify this drop in numbers.

The efficiency of blunt-end ligation may be increased by adding PEG to a concentration of 5% in the ligation buffer.

Always do the controls of ligation and transformation (tubes 4, 5, and 6) to try to pinpoint the source of any problems.

Procedure 8: Competent Cells

Recipes

TFB:
10 m*M* MES
100 m*M* rubidium chloride (RbCl)
45 m*M* manganese chloride ($MnCl_2 \cdot 4H_2O$)
10 m*M* calcium chloride ($CaCl_2 \cdot 2H_2O$)
3 m*M* hexaminecobaltic chloride

DTT/KAc:
2.25 *M* DTT
40 m*M* potassium acetate (pH 6.0)

2× YT (1 liter):
10 g Bacto tryptone
10 g yeast extract
5 g sodium chloride

High-Efficiency Cells

1. Toothpick an individual colony from the streaked plate of JM cells into ~10 ml of 2× YT and grow with shaking at 37° overnight.
2. To 30 ml of 2× YT add 0.3 ml of overnight culture and grow with shaking for around 2 hr to an OD at 600 nm of 0.4–0.6.
3. Transfer culture to a Falcon 50-ml disposable centrifuge tube and centrifuge at 2000 rpm for 10 min at 4°.
4. Pour off the 2× YT and drain the pellet well. Resuspend the cells very gently in 2.5 ml of cold TFB and incubate on ice for 15 min.
5. Add 100 μl of DMSO and incubate for 5 min on ice.
6. Add 100 μl of DTT/KAc and incubate for 10 min on ice.
7. Add 100 μl of DMSO and incubate for 5 min on ice.
8. Keep on ice and use on the day of preparation.

The high-efficiency transformation cells yield up to 100 times the number of recombinants normally obtained when using calcium chloride. Sub-

stituting the cheaper KCl for RbCl and the less malodorous DMF for DMSO gives a yield of recombinants approximately 10 times greater than when using calcium chloride.

When using high-efficiency competent cells, it is particularly important to use phosphatase-treated vectors, otherwise the plate is overcrowded with blue plaques and it is almost impossible to pick the recombinant plaques.

Since the cells need to be treated very gently, do not wash and centrifuge the cell pellet in TFB.

Procedure 9: Transformation

Recipes

Top agar (1 liter):
10 g Bacto tryptone
8 g sodium chloride
8 g Bacto agar

TYE agar (1 liter):
1 liter 2× YT
15 g Bacto agar

Xgal:
2% 5-bromo-4-chloro-3-indolyl-β-galactoside in DMF

IPTG:
2.5% isopropyl-β-D-thiogalactopyranoside in water

1. To a sterile glass culture tube on ice add
200 μl competent cells
20 μl fragment/vector ligation
Incubate on ice for 45 min with occasional agitation.
2. To a sterile glass culture tube in a heat block at 45° add
3 ml molten top agar
25 μl 2% Xgal in DMF
25 μl 2.5% IPTG in water
3. Heat shock competent cells/ligation mix at 42° in a waterbath for less than 5 min.
4. Quickly add the molten agar to the competent cells.
5. Without delay, pour the agar onto a TYE plate and swirl to distribute it evenly.
6. Allow 15 min for the agar to set and place the plate, inverted, in an oven at 37° overnight.

The duration of the heat shock at 42° will depend upon the type of tubes used, the optimum varying between 90 sec and 5 min. Pour the warm agar quickly onto the ligation mix to ensure good mixing and plate out immediately. If the agar has begun to set, the plate will have a rough uneven surface.

Freshly made agar plates can have a very high moisture content. If they are not dried at 37° overnight or stored at room temperature for a day or two before use they will "sweat," causing streaking of the plaques.

Phage Growth and DNA Isolation

The plaques obtained on the plate are areas of reduced growth resulting from phage infection of a bacterial lawn. Each plaque arises from a single transformation event and therefore each colorless plaque is an infection with a unique recombinant phage. These infection plaques can be used to seed individual small-volume cultures and the phage isolated from these cultures can be used to prepare single-stranded specific recombinant phage DNA.

Phage particles which are extruded from the infected host cells into the surrounding medium can be separated from the cells by centrifugation and concentrated by precipitation in polyethylene glycol. Single-stranded template DNA can then be purified from the phage by phenol extraction followed by ethanol precipitation. The purity of this template DNA is crucial to the quality of sequence obtained.

Procedure 10: Template Preparation

Recipes

2× YT (1 liter):
10 g Bacto tryptone
10 g yeast extract
5 g sodium chloride

20% PEG:
20 g polyethylene glycol (8000 mol. wt.)
14.6 g sodium chloride
Bring volume to 100 ml with water.

1. Toothpick an individual colony from the streaked plate of JM cells into ~10 ml of 2× YT and grow with shaking at 37° overnight.
2. Dilute enough of the overnight culture 1/100 with 2× YT to give sufficient volume for 1.5 ml medium for each plaque.

3. To sterile culture tubes (>10-ml capacity) add 1.5 ml of the diluted culture and toothpick a separate single plaque into each. Do not leave the toothpick in the tube.

4. Grow with vigorous shaking (>300 rpm) at 37° for 4.5–5.5 hr.

5. Transfer the cultures to 1.5-ml microfuge tubes and centrifuge for 5 min in a microfuge.

6. Transfer the supernatant to a clean microfuge tube, taking care not to carry over any cells, and add 200 μl of PEG solution.

7. Vortex the supernatant well and incubate at room temperature for at least 10 min.

8. Centrifuge the supernatant/PEG in a microfuge for 5 min and remove the PEG.

9. Respin the tube for around 30 sec and remove all residual PEG.

10. Resuspend the phage pellet in 100 μl of TE.

11. Add an equal volume of buffer-saturated phenol, vortex well, and leave for 5 min. Vortex and centrifuge 2 min in a microfuge.

12. Remove upper aqueous layer and add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of ethanol, and precipitate in dry ice/isopropanol for 20 min.

13. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.

14. Add 1 ml of 95% ethanol, centrifuge for 5 min, and pour off the ethanol.

15. Vacuum dry the pellet, redissolve in 30 μl of TE, and store at −20°.

Having obtained plaques on an agar plate, it is best to grow the phages and purify the DNA as soon as possible. Even after only a few days' storage at 4°, there can be a marked decrease in quality of the final sequence, since the background and incidence of artifact bands both increase.

Phage cultures should not be grown at temperatures greater than 37°, as the yields of M13 phages drop quickly with increasing temperature despite continued host growth.

The total growth period of 4.5–5.5 hr is also important. Overgrowth results in cell lysis and the host chromosomal DNA contributes to the general background. When transferring the phage supernatant from the cell pellet, take care not to disturb the pellet.

Ensure complete removal of the PEG after the phage precipitation and resuspend the phage before adding the phenol, as the pellet will be very difficult to redissolve in the presence of phenol.

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Dideoxy Sequencing

In the presence of the four deoxynucleotides (dNTPs), the Klenow fragment of DNA *Pol*I can extend a primer DNA molecule across a single-stranded template DNA. The dideoxynucleotide analogs (ddNTPs) lack the 3′-OH necessary for chain extension and, if they are incorporated into a growing DNA molecule, no further nucleotides can be added. So, if the chain extension is done in the presence of the four-dNTP mixture, which also contains a small proportion of a specific ddNTP, the dideoxy analog will compete with the dNTP analog and will be incorporated randomly, producing a range of different length primer-extended chains, each ending with the specific ddNTP, on a single-stranded template DNA. Four separate reactions can be performed, each with a different ddNTP, so that chains terminating at every position in the target sequence are represented within the four reactions. After denaturation from the template strand, these four sets of reaction products can be fractionated on a polyacrylamide gel, and, by using one or more radioactively labeled dNTPs, can be visualized by autoradiography on X-ray films. A ladder pattern results, where each successive band from the bottom to the top of the gel represents a difference in length of one nucleotide and the track in which it appears indicates the nucleotide type. The sequence of nucleotides beyond the position of primer hybridization can be read simply by noting the order of bands from the bottom to the top of the four nucleotide-specific tracks.

The improvements in DNA sequence technology seen in recent years result from more reliable commercial products, a switch in the radiolabel used, and a simplification of the basic manipulations with a result of maximizing output but at the same time minimizing the effort required.

An increase in the use of DNA sequence analysis as a basic tool in all areas of molecular biology has ensured a good market for commercial suppliers. This and the inherent competition has done much to improve overall quality of products, particularly of the Klenow fragment DNA *Pol*I.

Using ^{35}S-labeled nucleotides has several distinct advantages over using ^{32}P as label:

1. The low-energy particles produced by ^{35}S decay result in less "spread" than when using ^{32}P. Consequently the bands are much sharper.
2. The lower energy of ^{35}S also reduces the damage from radiolysis. Radioactive decay of ^{32}P-labeled nucleotides, incorporated into DNA molecules, breaks the phosphate backbone. This produces fragments with

nonspecific 5′ ends which give a dark background smear on sequence autoradiographs.

3. The energy of ^{35}S emission is around 10% that of ^{32}P and, therefore, there is a considerable safety advantage to using ^{35}S.

4. The half-life of ^{35}S is around six times longer than that of ^{32}P, so very little is wasted from it getting too "cold."

All reactions are at present carried out in disposable microtiter trays as shown in Fig. 2. Each tray is used as the vessel for up to 24 different

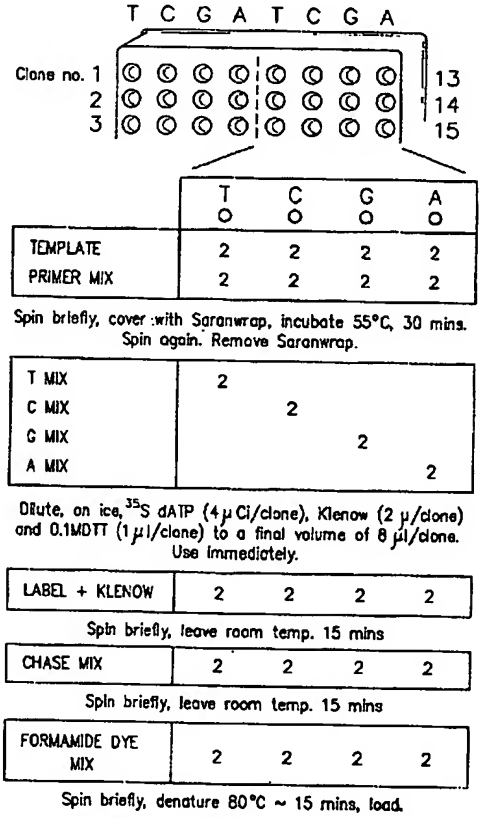

FIG. 2. A schematic diagram showing the order of addition of the reagents for a set of sequencing reactions on a single clone carried out in a microtiter tray. Volumes are given in microliters.

background smear on sequence

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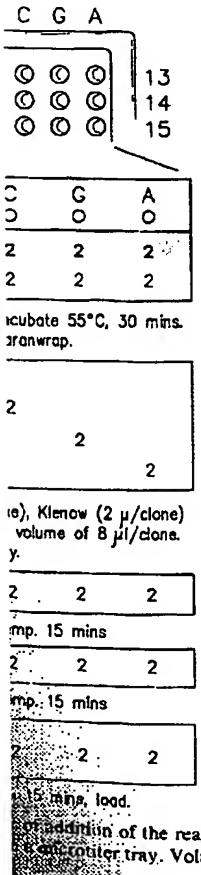

of addition of the reagents for a set of
96-microtiter tray. Volumes are given in

sequence reactions (96 individual chain extensions). This makes the manipulations involved in large-scale sequencing projects much easier. Reagent additions are performed, wherever possible, using repetitive dispensers (Hamilton PB600 fitted with 1710 LT syringe). This not only means that many reactions can be performed simultaneously, but also reagents such as diluted enzyme can be aliquotted very quickly. Although these dispensers are less accurate than most single-shot pipets, there is no detectable difference in the end product. Reagents are pipetted as separate droplets on the side of the wells and are mixed by very brief centrifugation in a bench-top centrifuge in a suitable rotor (I.E.C. Centra 4X).

Procedure 11: Sequence Reactions

Recipes

TE:
10 m*M* Tris (pH 8.0–8.5)
0.1 m*M* EDTA (Na_2)

10 m*M* ddNTPs in TE:
ddTTP, 6.1 mg/ml
ddCTP, 5.8 mg/ml
ddGTP, 6.2 mg/ml
ddATP, 6.2 mg/ml

50 m*M* dNTPs in TE:
dTTP, 31.2 mg/ml
dCTP, 29.6 mg/ml
dGTP, 31.6 mg/ml
dATP, 29.5 mg/ml

0.5 m*M* dNTPs:
1 : 100 dilution of 50 m*M* stocks in TE

dNTP chase:
0.5 m*M* dTTP
0.5 m*M* dCTP
0.5 m*M* dGTP
0.5 m*M* dATP
(From 50 m*M* stocks in TE.)

TM:
100 m*M* Tris (pH 8.5)
50 m*M* $MgCl_2$

NTP mixes:

	T	C	G	A	
0.5 m*M* dTTP	25	500	500	500	
0.5 m*M* dCTP	500	25	500	500	
0.5 m*M* dGTP	500	500	25	500	
10.0 m*M* ddTTP	50	—	—	—	
10.0 m*M* ddCTP	—	8	—	—	
10.0 m*M* ddGTP	—	—	16	—	
10.0 m*M* ddATP	—	—	—	1 (3 when using [^{32}P]dATP)	
TE	1000	1000	1000	500	

Formamide dye mix:
100 ml formamide (deionized with mixed-bed resin)
0.1 g xylene cyanole FF
0.1 g bromphenol blue
2 ml 0.5 *M* EDTA (Na_2) (pH 8.0)

10× TBE (1 liter):
108 g Tris
55 g boric acid
9.3 g EDTA (Na_2)
Bring volume to 1 liter with deionized water.

1. For each template to be sequenced, to a 1.5-ml microfuge tube add
1 μl primer (0.2 pmol) (Collaborative Research)
1 μl TM
7 μl water
Scale this up by the number of templates to be sequenced.
2. Using a disposable microtiter tray such as Falcon 3911, assign four wells as T, C, G, and A for each template.
3. To each well add 2 μl of primer/TM mix.
4. To each of the wells T, C, G, and A add 2 μl of the appropriate template DNA.
5. Cover the wells with a layer of Saran wrap.
6. Centrifuge the tray briefly to mix the reagents and incubate in an oven at 55° for at least 30 min.
7. Centrifuge the tray to concentrate any condensation and remove the Saran wrap.
8. Into each well dispense, close to the rim, 2 μl of the appropriate NTP mix (i.e., add T mix to T well).

A
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1 (3 when using [^{32}P]dATP)
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9. Wearing gloves, dilute 4 μCi of [^{35}S]dATP (for each template to be sequenced) and 1 μl 0.1 *M* DTT/clone in a small silanized glass tube to 0.5 μCi/μl with water and place on ice.

10. To the diluted [^{35}S]dATP add Klenow fragment DNA *Pol*I (Boehringer Mannheim) to 0.125 U/μl, mixing thoroughly.

11. WITHOUT DELAY, dispense 2 μl of ^{35}S/Klenow to each well, taking care not to touch the drops of NTP mix, and centrifuge as briefly as possible (maximum acceleration to 2000 rpm and stop) to mix. Incubate at room temperature for 15 min.

12. Add to each well 2 μl of 0.5 m*M* dNTP chase solution and centrifuge very briefly to mix. Incubate at room temperature for 15 min.

13. Add to each well 2 μl of formamide dye and centrifuge to mix.

14. Heat denature the reactions uncovered, by incubating the tray in an oven at 80° for 15 min immediately before loading.

The majority of problems associated with sequence reactions are attributable to either poor template quality or to the Klenow DNA *Pol*I. The enzyme needs to be treated very gently and kept cold as often as possible, especially when diluted. There is a frequent tendency to be miserly with the amount of enzyme used, but this can be false economy if the sequence reactions have to be repeated because of inadequate results. The enzyme activity quoted by suppliers can sometimes bear little relationship to the quantity needed and each newly acquired batch is best titrated to optimize the system. By and large around 1–2 units per template will be required for good results. Typical signs of insufficient or inadequate enzyme are sequence-specific terminations in all four lanes, particularly toward the top of the gel. In severe cases these common bands will occur further down the gel. In mild cases the incidence of track and sequence-specific artifacts increases.

Problems caused by secondary structure within the template during the reaction are quite rare, but can produce an almost complete block to chain extension, beyond which the incorporation drops to near zero. For some situations, performing the sequence reactions at elevated temperatures will help. In these very rare situations using reverse transcriptase may possibly be of use, but it is of no benefit for routine work.

A good investment may be a commercial sequence kit, as this will provide a control system which can be used as a troubleshooting guide. Substitution of each reagent in turn into the control will quickly identify the source of any problem. If the kit does not work either, the supplier should be able to give advice and assistance.

The compositions of the nucleotide mixes quoted are for use with

radioactively labeled dATP, cold dATP being eliminated. Other radiolabeled nucleotides can be used by altering these solutions to exclude the appropriate cold nucleotide. The volumes described should be used as a guide to get started but will probably need further adjustment to give the best possible results. Increasing the volume of dideoxynucleotide solution in the mixture will decrease the average length of chain, making the lower bands corresponding to the shorter DNA molecules darker. Increasing the relative quantity of deoxynucleotide will have the opposite effect, making the upper bands darker. If the ratios do have to be altered, a change of around 100% will be needed to make a significant difference. Once the ratio of deoxy to dideoxy has been properly titrated, the combined solution can be stored in aliquots. These solutions will keep for a long time at −20°.

Using combined solutions to reduce the number of total additions can be taken a step further by combining the nucleotide mixes with the primer prior to annealing, with no detectable inhibition of primer–template hybridization or degradation of the nucleotide mixes.

All the various items in the reactions can be fine-tuned. For example, take a template of average DNA concentration (as assayed on a minigel) and optimize the amount of primer for this average DNA concentration. This primer concentration can be used routinely for all sequence reactions. The darkness of bands after an overnight exposure can be altered by scaling the primer–template concentration.

The denaturation step is carried out at 80°, as above this temperature the plastic of the microtiter plates begins to melt. The duration will depend upon the precise temperature of the incubator and is the time required to reduce the volume sufficiently so that the entire sample can be loaded onto a single track on the gel.

Sequence reactions using [^{35}S]dATP can be stored at −20°, before running on a gel, and will still give good results provided the formamide dye mix is omitted and added just before denaturation. The only major deterioration is a reduction in intensity due to radioactive decay and will take several weeks. The presence of formamide may cause degradation, making the results unacceptable. It is also possible to store the annealed primer–template mix at −20° and to continue with the sequence reactions at any time up to several weeks afterwards with no ill effect.

Acrylamide Gels

The logarithmic relationship between the length of a DNA fragment and its mobility on polyacrylamide gels produces a very large spacing between the consecutive bands of a sequence ladder at the bottom of the

ing eliminated. Other radiola-
these solutions to exclude the
described should be used as a
further adjustment to give the
of dideoxynucleotide solution
gth of chain, making the lower
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roduces a very large spacing
e ladder at the bottom of the

gel, but an ever-decreasing spacing at the top of the gel. By increasing the salt concentration of the buffer within the gel toward the bottom, a potential difference gradient can be set up which has a lower voltage drop over the bottom part of the gel.[8] This retards the smaller, faster moving fragments, not only evening out the spacing between consecutive bands up the gel, but resulting in the fractionation of the larger fragments through a greater length of gel, thereby increasing its resolving power. In practice, the best compromise between maximum resolution and decreasing band spacing, which makes reading difficult, is found to be a gradient over the lower third of the gel. The use of buffer gradient polyacrylamide gels has increased the sequence information obtainable from each gel by over 30%. What makes the buffer gradient gels so practical is the simple way in which they are produced. The gel plates and format are conventional, and the gradient is formed by limited mixing of two solutions in a pipet before pouring in the usual way.

The same field-strength gradient can be created by altering the gel thickness along its length, using multiple thickness spacers at the bottom.[9] These "wedge" gels suffer from a few drawbacks. If the thickness at the bottom is greater than about 0.8 mm, the gel takes much longer to dry and there is an unacceptable loss of resolution. The conventional thickness of sequencing gels is 0.35 mm, which combines high resolution with ease of handling. Thinner plastic can be used to increase the wedge gradient, but loading becomes difficult and tedious, especially if many samples are being applied. The limitation on thickness limits the magnitude of the voltage gradient and reduces the usefulness of wedge gels, especially for longer gel lengths. Without distorting the glass plates, the shape of the gradient is also inflexible. However, the principle of the wedge gel can be usefully applied to agarose gels by setting and running the gel at an angle.

To increase further the amount of information obtainable from a sequencing gel, the length of the gel can be increased, typically by using 50-cm instead of 40-cm-long plates. Although in theory it is possible to use much longer gels, the advantages in terms of increased sequence data available are outweighed by the time needed to run the gels, to say nothing of the problems involved in their pouring and manipulation and the limitations imposed by X-ray film sizes.

Probably the simplest means of increasing the sequence information per gel is reducing the gel slot size to a minimum. It is possible to load 72 samples (18 sequence reactions) or more on a single 20-cm-wide gel. Normally we load 40 samples on a 20 × 50-cm buffer gradient gel.

[8] M. D. Biggin, T. J. Gibson, and G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963 (1983).

[9] A. T. Bankier and B. G. Barrell, *Tech. Life Sci.: Biochem.* **B508**, 1 (1983).

Procedure 12: Buffer Gradient Gels

Recipes

10× TBE (1 liter):
108 g Tris
55 g boric acid
9.3 g EDTA (Na_2)
Bring volume to 1 liter with deionized water.

40% acrylamide (1 liter):
380 g acrylamide
20 g *N,N'*-methylene bisacrylamide
Bring volume to 1 liter with deionized water; stir gently with 20 g mixed-bed resin (e.g., Amberlite MB 1); filter through sintered glass to remove resin; store at 4°.

0.5× TBE 6% gel mix (500 ml):
75 ml 40% acrylamide
25 ml 10× TBE
230 g urea (B.R.L. ultrapure)
Bring volume to 500 ml with deionized water and filter through sintered glass; can be stored for 3–4 weeks at 4°.

5.0× TBE 6% gel mix (200 ml):
30 ml 40% acrylamide
100 ml 10× TBE
92 g urea (B.R.L. ultrapure)
10 mg bromphenol blue
Bring volume to 200 ml with deionized water and filter through sintered glass; can be stored for 3–4 weeks at 4°.

25% AMPS:
25% ammonium persulfate in water.
Can be stored for several months at 4°.

TEMED (*N,N,N',N'*-tetramethyl-1,2,-diaminoethane)

1. Carefully clean one pair of glass gel plates.
2. Wearing gloves and working in a fume hood, silanize the smaller or eared plate by spreading ~2 ml of silanizing solution (2% dimethyldichlorosilane in trichloroethane) over the inner surface of the glass plate with a tissue. Allow to dry.
3. After wiping clean with 95% ethanol, assemble the glass plates using 0.5-cm-wide 0.35-mm Plastikard (Slaters Plastikard, Matlock Bath,

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MB 1); filter through sintered

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inner surface of the glass plate
assemble the glass plates
Plastikard, Matlock Bath,

United Kingdom) side spacers and 25-mm polyester tape (code 491Y, Neil Turner and Co., Watlington, Kings Lynn, United Kingdom).

4. For a 40 × 20-cm gel, into two separate beakers put 7 ml 5× TBE gel mix and 30 ml 0.5× TBE gel mix.

5. Initiate polymerization by first adding 14 μl (to the 5×) and 60 μl (to the 0.5×) of 25% ammonium persulfate and then adding 14 μl (5×) and 60 μl (0.5×) of TEMED.

6. Take up into a 50-ml plastic syringe ~22 ml of the 0.5× TBE gel mix.

7. Using a pipet controller, first take up into a 10-ml pipet 5 ml of 0.5× TBE gel mix and then 5 ml 5× TBE gel mix. Form a rough gradient by allowing 2–3 air bubbles to pass up the pipet.

8. Pour the gradient down the inner edge of the gel plates, holding them at an angle of about 45°. Add the gel mix slowly and try to maintain an even flow. When the pipet is empty, lower the plates to horizontal to stop the flow, while the syringe is being picked up. It is extremely important to maintain a continuous flow of gel mix, so try to continue on to the next step as quickly as possible.

9. Add the remaining 0.5× TBE gel mix from the syringe to fill the glass plates.

10. Put the slot former in place and clamp the edges of the gel plates with foldback clips (only over side spacers) and allow the gel at least 30 min to polymerize.

11. Wash out the syringe and pipet before the gel sets (they can be reused) but keep the rest of the 0.5× TBE gel mix for topping up if necessary.

12. If storing the polymerized gel overnight, cover the top of the gel with Saran wrap to prevent drying out.

For a 50 × 20-cm gel, the procedure for pouring the gradient is identical, except that 30 ml of 0.5× TBE gel mix should be taken up in the plastic syringe, and the gradient is formed by mixing 6 ml of 0.5× TBE gel mix with 6 ml of 5× TBE gel mix in a 25-ml pipet.

Ensure the glass plates are scrupulously clean to avoid air bubbles when pouring the gel. Keep the flow as smooth as possible, as interruptions will introduce air bubbles. Often if the bubbles are few, they can be removed or pushed to the side with a length of emulsion-stripped X-ray film (soak undeveloped film in hot water). If the bubbles are in the gradient part of the gel or there are many of them, it is often easier to pour another gel.

Try to pour the gradient gel mix as a 5-cm wide flow. This ensures that

any severe irregularities of the gradient down the pouring edge are outside the area used for running samples. When topping up with the syringe, move the syringe along the top as the plates fill. This helps stop "kick-back" of the gradient mix up the other edge of the plates. Do not worry about irregularities in the gradient, as they seldom pose any serious problem to reading the autoradiograph.

Polymerization time can be increased by decreasing the volume of the catalyst TEMED which is added. This may be a good idea for the first few attempts until confidence is gained.

Procedure 13: Gel Running

1. Remove the slot former carefully from the gel and wash the slots with deionized water to remove any unpolymerized acrylamide.
2. Using foldback clips clamp the gel to the electrophoresis apparatus and fill the top and bottom tanks with 1.0× TBE.
3. Using a Pasteur pipet, flush the slots immediately and again just before loading.
4. With a drawn-out length of polypropylene tubing or glass capillary and a mouthpiece, load 1.5–2.0 μl of each sample onto the gel, noting the order of the clones. The gel must be completely loaded within 30 min, otherwise some renaturation of samples will occur.
5. Connect the apparatus to a power supply (positive to the bottom) and run at constant power of around 35–40 W until the bromphenol blue marker is just off the bottom of the gel (~2.5 hr for a 40-cm gel, ~3 hr for a 50-cm gel).
6. Disconnect the power, drain off the buffer, take the gel/plates from the apparatus, and completely remove the polyester tape.
7. Pry apart the glass plates gently, using a spatula, trying to keep the gel stuck to the larger, or noneared, plate.
8. Slowly immerse the gel/plate in 10% acetic acid in water and leave for at least 15 min.
9. Trying to keep the gel stuck to the glass plate, remove the gel/plate from the acetic acid and drain well.
10. Transfer the gel to 3-mm paper by placing the paper on the gel; after applying gentle pressure over the whole area of the gel, gently peel the paper/gel off the glass plate. For a 50-cm gel, it is necessary to trim off the top ~8 cm, to enable the gel to fit a standard film cassette. The band resolution is in any case so poor at the top of the gel that there is no loss of sequence information involved.
11. Cover the gel with Saran wrap, trim the edges to fit the gel drier, and completely dry the gel at 80° under vacuum.

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-cm gel, it is necessary to trim off
standard film cassette. The band
p of the gel that there is no loss of

rim the edges to fit the gel drier,
vacuum.

12. Peel off the Saran wrap and label the gel with radioactive ink. Place the dried gel in a film cassette in direct contact with X-ray film and leave overnight at room temperature.

Ensure adequate fixing, as this dialyzes out the urea, which otherwise prevents proper drying and results in sticking of the gel to X-ray films.

When using ^{35}S, the Saran wrap must be removed, as the low-energy particles of ^{35}S decay will not effectively pass through it.

If, on opening the gel plates, the gel tends to stick to both plates, pry them apart while submerged in the acetic acid. After fixing, position the gel centrally on the glass plate and remove the gel/plate with the aid of a piece of plastic netting to prevent the gel escaping. The gel can then be drained, carefully transferred to paper, and dried as normal.

Although the four samples of the sequence reaction can be loaded in any order, it is best to have the G and C tracks adjacent as they are the most likely source of anomalous mobility problems. It is easier to determine which band comes next when they are close together. If they are placed in the center of the set of four, reading the complementary sequence is facilitated by flipping over the film and reading top to bottom.

Reading Dideoxy Sequences

Reading sequence autoradiographs accurately is a skill which only comes with practice and experience. The best way to gain this experience is by comparing gel readings with a previously determined sequence, such as that of M13, and reconciling any differences between the two. When reading dideoxy sequences, there are a few guidelines which may help where problems or ambiguities arise.

General

1. Mark out the four tracks of the sequence to avoid confusion with tracks from other sequences.
2. Identify the ends of the insert; this avoids hours spent in reading vector sequences by mistake.
3. It is equally important to "read" the band spacings as well as the bands themselves. The spacing can tell you how many bands to expect between two points and can highlight fragments running anomalously.

Band Intensity

1. Single C bands are generally weak.
2. In a run of C bands, the first is normally very weak, and the second

is strongest. Occasionally, when preceded by a G, the first band can be of normal intensity.

3. Weak G bands are sometimes seen when preceded by a T.
4. In a run of A bands, the first is often stronger.

Artifacts

Artifact bands are almost always indicative of a system not working properly, and under ideal conditions they should pose no problem.

1. Extra C band between T and G, most often in sequence TGCC.
2. Extra C and T bands opposite A, most often in the sequence GCA.
3. Artifact A band preceding real A. This most probably results from an inadequate chase step due to the enzyme failing. The chains terminate not because of incorporation of a dideoxy, but because the rate of [^{35}S]dATP incorporation is limited both by its low concentration and by the reduced efficiency with which it is used as a substrate by DNA *Pol*I.
4. A series of very dark "pile-up" bands, across all four tracks, most probably result from failure of the polymerase to traverse a region of conformation change in the template, e.g., base stacking of G nucleotides. These pile-up bands can often be remedied by lowering the salt concentration of the reaction buffer, as this destabilizes the base interactions.
5. Shadow A bands in other tracks. This is specific to using ^{35}S-labeled dATP. It is an indication of poor polymerase activity. The bands most probably arise from the 3′ → 5′ exonuclease activity of the enzyme. As thionucleotides are resistant to the exonuclease, chains ending with a thionucleotide will accumulate. If the polymerase is working well, this is not a problem.

Anomalous Band Spacing

1. Irregularities in the gradient can produce minor band spacing anomalies on the gel.
2. "Smiling" of the bands across the gel results from the difference in heat losses between the center and the edge of the gel. This smiling can be greatly reduced by avoiding the very edge of the gel, or by clamping an aluminum plate to the front of the gel plates. Gradient gels, because of their retarding effect, also tend to reduce the smiling effect.
3. If even a very short stretch of complementarity occurs at the end of a fragment, the denaturation conditions used may fail to prevent the fragment from folding back on itself. Such fragments containing secondary structures run with anomalous mobility on gels, appearing to migrate at the same rate as shorter single-stranded fragments. This is seen on autora-

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diographs as a "compression," a region of narrow band spacing, often with bands from different tracks running at the same position, followed by a region of unusually wide spacing. Compressions can be the most serious problem encountered when DNA sequencing, as bands can be missed completely. For this reason, it is very important that the sequence be determined entirely from both strands (the compression occurs in a different place on the other strand).

Completing a Project

Using the random method, the DNA sequence is accumulated very rapidly at the beginning of a project, but later, much of the information is confirmation of the data already obtained. Although a contiguous sequence may be gained quite quickly, this does not mark the end of the project. The possibility of local base pairing causing gel interpretation problems makes it extremely important that sequences be determined entirely from both strands. Sequencing of randomly generated clones should be continued until the complete sequence of both strands is obtained. In practice, this has been found to be when the total sequence accumulated is around six times the original fragment length. There is often a tendency to stop random sequencing too early under the misapprehension that the sequence is sufficiently complete, or that it could be completed more quickly by other means. This is rarely true. Sequencing a few more clones takes much less time, at most a few days, than planning a strategy, cloning specific regions for sequencing, or even reversing the orientation of inserts.

Failure to obtain a single contiguous sequence by the time a sixfold redundancy is reached may be due to one of several reasons.

1. If the host cells used for the M13 cloning have an *Eco*K restriction system (e.g., JM101) and the original fragment was not isolated from a modifying host, subfragments containing the *Eco*K recognition sequence will only be cloned very rarely.
2. Some sequences, often highly repetitive, clone very rarely or are deleted readily from M13.
3. If the original fragment is not self-ligated, the subfragments generated by sonication do not have a random distribution. This results in some areas (e.g., the ends) being represented many times while other regions are seldom represented at all.
4. The ends of fragments may be underrepresented in the final sequence, since potential inverted repeats, formed during self-ligation, are rarely seen cloned in M13.

5. Stray sequences, usually of unknown origin, can find their way into the sequence database. These will contribute to the total number of "contigs" (contiguous sequences) but should be easily identified at the end of the project.

Double Stranding

If, after sequencing a total greater than six times the fragment length, a complete double-stranded sequence has not been gained, the sequences of particular regions can be gained by more specific means.

The first thing to try is extending existing gel readings. On average each clone is read out to 250–300 nucleotides. It may be possible, using longer electrophoresis times, to read sequences greater than 400. Gradient gels are not very suitable for such extended gel running times as the spacing between consecutive bands becomes unacceptably narrow. Standard 1× TBE gels of between 4 and 6% acrylamide give much better results.

Clones containing the sequence spanning a gap can be identified by hybridization, using M13 clones from both sides of the gap as probes. Or a specific restriction enzyme fragment spanning a gap can be predicted from the sequence and a cloning experiment performed using a vector tailored to accept this particular fragment.

Reversing the orientation of a cloned insert is a method of gaining sequence data from the other strand of a particular region. This is most easily carried out by utilizing pairs of complementary vectors, e.g., mp8/9 or mp10/11, where the polylinker regions are inverted with respect to each other.[10] An insert can be cut out of a vector using two flanking restriction enzyme sites, one on each side of the insert. The excised fragment can be recloned into the complementary vector cut with the same two restriction enzymes. Because the order of the restriction sites is reversed between these vectors, the orientation of the insert will be reversed.

Procedure 14: Clone Reversal

Recipes

TE:
10 m*M* Tris (pH 8.0–8.5)
0.1 m*M* EDTA (Na_2)

TM:
100 m*M* Tris (pH 8.5)
50 m*M* magnesium chloride

[10] J. Messing and J. Vieira, *Gene* **19**, 269 (1982).

dNTP chase:
0.5 m*M* dTTP
0.5 m*M* dCTP
0.5 m*M* dGTP
0.5 m*M* dATP
(From 50 m*M* stocks in TE.)

1.0% LGT gel mix:
25 ml 1.0× TBE
0.25 g LGT agarose
Dissolve at 100° and add 1 μl of 10 mg/ml ethidium bromide just before pouring.

TBE dye mix (100 ml):
10 ml 10× TBE
0.1 g bromphenol blue
20 g sucrose
Bring volume to 100 ml with water.

10× ligase buffer:
500 m*M* Tris (pH 7.5)
100 m*M* $MgCl_2$
100 m*M* DTT

1. To a 1.5-ml microfuge tube add
2 μl primer (0.4 pmol)
1 μl TM
10 μl template DNA (from a standard preparation)
2. Centrifuge briefly in a microfuge to mix and incubate the tube in an oven at 55° for at least 45 min.
3. Add 2 μl of chase mix and 2 units of Klenow fragment DNA *Pol*I and centrifuge very briefly to mix.
4. Leave at room temperature for 10–15 min.
5. Place the tube in a 70° waterbath for 15 min to denature the enzyme.
6. Add 2 μl of 10× appropriate restriction enzyme buffer and 5 units of each of two suitable restriction enzymes (e.g., *Bam*HI and *Eco*RI).
7. Put tube in 37° waterbath or oven for 2 hr.
8. Pour a 25-ml 1% LGT agarose minigel (2-cm slots) and place at 4° for at least 1 hr to set.
9. To the restricted DNA from step 7 add 5 μl of TBE dye and load onto the minigel.
10. Connect the minigel to a power supply and run at 30 mA for around 30 min, or until the fragment band is resolved from the vector band.

11. Cut out the fragment band using a scalpel or a safety razor blade and put the gel slice into a 1.5-ml microfuge tube.
12. Put the tube in a 70° waterbath or heat block to melt the gel slice.
13. Add an equal volume of buffer-saturated phenol. Vortex well and leave for 5 min. Vortex and centrifuge 2 min in a microfuge.
14. Remove upper aqueous layer and re-extract phenol phase with an equal volume of TE.
15. Combine the aqueous phases and phenol extract a further two or three times or until a clear interface is obtained.
16. Add 1/10 volume of 3 *M* sodium acetate, 2.5 volumes of ethanol, and precipitate in dry ice/isopropanol for 20 min.
17. Centrifuge for 5 min in a microfuge and carefully pour off the ethanol.
18. Add 1 ml of 95% ethanol, centrifuge for 5 min, and pour off ethanol.
19. Vacuum dry the pellet and redissolve in 20 μl of TE.
20. To a 1.5-ml microfuge tube add:
    1 μl 10× ligase buffer
    1 μl 10 m*M* rATP
    5 μl fragment (from step 19 above)
    1 μl cut vector [cut with the same enzyme(s)]
    20 U T4 DNA ligase
    Incubate tube at room temperature for 2–3 hr.
21. Transfect DNA into competent cells.
22. Prepare template DNA from at least two recombinants.
23. Sequence the templates as normal.

Ensure an adequate heat-kill step, as the Klenow polymerase will otherwise fill in the ends of the fragment, preventing ligation to the vector.

Before starting a clone-reversal experiment, check that the original clone insert is of a suitable length; will it be possible to sequence across the area of interest from the other side of the insert? For insert fragments of the normal length (300 bp), the band on the agarose gel can be expected to run just above the blue marker dye. For shorter inserts, the blue marker dye can sometimes obscure the band to be cut out. Loading the sample in 25% sucrose, 1× TBE will avoid this problem.

An alternative to the time-consuming recloning involved in clone reversal is reverse sequencing.[11] The nascent strand of a primer extension can be used as a template for a synthetic oligonucleotide primer on the opposite side of an insert. If this reverse primer is used in a dideoxy

[11] G. F. Hong, *Biosci. Rep.* **1**, 243 (1981).

sequence reaction, the sequence of the opposite strand can be determined. As this is, in essence, sequencing on a double-stranded DNA template, as might be expected, the quality is not comparable with that of a normal single-stranded template sequence.

Compressions

A common reason for inability to complete a sequence is the ambiguity produced by local secondary structure in the chain extension products, often described as a compression because of the anomalous narrow band spacing on sequence autoradiographs. These are artifacts of the electrophoresis, not of the chain extension reaction. Even quite short stretches of complementarity, as few as 3 G–C bp with a small "loop" of 2–5 bases, can result in an unreadable sequence over a small local area on gels. The peculiar spacing is due to chains with short stretches of secondary structure at the 3′ end having the same mobility on polyacrylamide gels as shorter linear chains. At positions on the autoradiograph beyond the compression, the band spacing is unusually large, as the increasing chain lengths destabilize the small secondary structure, and the fragment mobilities revert to normal.

The sequence across these compressions can normally be resolved by sequencing both strands. The mobility artifact is only produced with chains of sufficient length for the base pairing to form. This means that the region of ambiguity will be displaced when sequenced on the other strand (the problem area is on the other side of the center of complementarity).

In cases where the possibilities for base pairing are more complex, a compression can cause gel artifacts at the same relative position when sequenced on either strand. These complex compressions can be extremely difficult to resolve, as the techniques available tend to give variable-quality results and are for this reason normally used only as a last resort.

The simplest way of destabilizing the short regions of base pairing which cause compressions on gels is to run the gels under more denaturing conditions, such as increasing the urea concentration in the gel mix, running the gel at elevated temperatures using a heated jacket, or by substituting formamide for some or all of the water in the gel mix. A substitution to a concentration of 25% formamide has been found to give the best reproducibility. Formamide gels have been most useful when the compression is fairly close to the priming site and the gel is 8 or 10% acrylamide to maximize the band spacing. The formamide should be deionized using a mixed-bed resin immediately before preparing the gel.

Deoxyinosine triphosphate can be substituted for dGTP in sequence

reactions with the advantage that I–C base pairs, having only two hydrogen bonds, are less stable than G–C base pairs. The only alterations required are a higher concentration of dITP and a lower ddGTP concentration, as inosine is less efficiently used as a substrate by DNA *Pol*I. Inosine mixes can be quite useful for some weaker secondary structures; however, when used on more complex compressions or very stable structures, the sequence results are generally much poorer than when using GTP mixes. An alternative is to use deoxy-7-deazaguanosine triphosphate, which has recently been reported to give better results.[12]

Procedure 15: Inosine Reactions

Recipes

ITP mixes:

	T	C	G	A	
0.5 m*M* dTTP	25	500	500	500	
0.5 m*M* dCTP	500	25	500	500	
2.0 m*M* dITP	500	500	25	500	
10 m*M* ddTTP	50	—	—	—	
10 m*M* ddCTP	—	8	—	—	
10 m*M* ddGTP	—	—	2	—	
10 m*M* ddATP	—	—	—	1 (3 when using [^{32}P]dATP)	
TE	1000	1000	1000	500	

1. Perform the sequence reactions as normal but using dITP mixes.
2. Use the standard 0.5 m*M* NTP chase mix.

Secondary structure G–C base pairing can be prevented completely by chemical modification of the cytosine residues. In one method, sequence reactions are carried out as normal and the chemical modification is done on the reaction products, changing C residues to 5,6-dihydro-6-sulfomethoxycytosine.[13] The modification reaction needs to be as close to 100% complete as possible, since varying numbers of unmodified C residues will cause the same length fragment to have varying mobility, resulting in multiple bands.

This technique seldom gives a perfect readable sequence, but if the modified sequence reactions are run on a high-percentage polyacrylamide

[12] S. Mizusawa, S. Nishimura, and F. Seela, *Nucleic Acids Res.* **14**, 1319 (1986).
[13] N. S. Ambartsumyan and A. M. Mazo, *FEBS Lett.* **114**, 265 (1980).

TROUBLESHOOTING GUIDE

Problem	Possible causes	Solution
	Procedures 1–10: Preparation of Templates	
(a) Very few or no recombinant phages after transformation	(i) Low-efficiency transformation: check *Alu*-cut λ control plate	Use only good growing cells in log phase. Ensure competent-cells are treated gently, and the DMF/DMSO is of good quality
	(ii) Insufficient or too much insert DNA in ligation	Repeat ligation using a wider range of insert-DNA concentration
	(iii) Faulty end repair has lowered effective concentration of DNA in ligation	Re-end repair, ensuring that $[Mg^{2+}] \geq 5$ m*M*.
(b) White recombinant plaques greatly outnumbered by blue vector plaques	(i) Large amount uncut vector present: check vector + ligase-negative control plate	Repeat vector preparation. Gel purify the linearized vector on 0.6% LGT agarose gel
	(ii) Phosphatase step has failed: check vector + ligase-positive control plate	Repeat phosphatase treatment in manufacturer's recommended buffer
	(iii) Vector cut with wrong restriction enzyme, preventing vector/fragment ligation	—
	(iv) On cloning some small, specific fragments which have an open reading frame, it is possible to generate "blue" recombinants	If suspected, grow and sequence a few blue plaques
(c) Many white plaques, when sequenced, do not contain inserts	(i) Vector preparation has resulted in loss of bases at cut site. Religated vector has frameshift in marker gene, and is no longer detectable	Repeat vector preparation. Check phosphatase for exonuclease activity (examine sequence of false whites for missing bases in polylinker). Rephosphatase in absence of Mg^{2+} to block exonuclease activity
	(ii) Too little or no Xgal	—
	(iii) Contamination of the transformation with a recombinant phage	Use good microbiological practice when handling phage supernatants

(continued)

TROUBLESHOOTING GUIDE (*continued*)

Problem	Possible causes	Solution
(d) No PEG pellet in template preparation procedure	(i) Cultures grown over temperature (≥38°)	Lower temperature to 36° on next attempt
	(ii) Old or incorrectly made PEG fails to precipitate phage	Add an extra 100 μl of PEG to cultures with no phage pellets and leave for 20 min. Spin 5 min. Make fresh PEG
(e) Large pellet still visible after phenol extraction and ethanol precipitation during template preparation procedure	Phage coats have not been removed in phenol extraction	Redissolve pellet in 50 μl TE and phenol extract again. Ethanol precipitate as normal. In future template preparation, ensure PEG pellet is completely dissolved by vigorous shaking before phenol is added
	Procedures 11–13: Sequencing	
(a) No radioactivity in any track	(i) No label or wrong label added	Ensure α- and not γ-labeled nucleotide has been used
	(ii) Primer or enzyme omitted	—
	(iii) Concentration of EDTA too high	Check TE. (Concentration of unchelated Mg^{2+} needs to be >5 m*M*)
(b) No sequence in set of four tracks	(i) No template DNA	When random sequencing it is often less time consuming to simply ignore these template preps
	(ii) Primer site deletion	Any deletion around the primer site usually includes the insert, and the template is best ignored
(c) No sequence in specific nucleotide track	(i) Incorrect NTP mix	Most probably no dNTP in mix, or possibly extremely high ddNTP concentration
	(ii) Specific NTP mix omitted	—
(d) No sequence in single track	Reagent omitted	—
(e) Very faint sequence in set of four tracks	(i) Low template concentration	Check concentration on an HGT agarose minigel. Often, very large insert clones need a longer growth time
	(ii) Template contains very few T residues	Very rare. Use an alternative α-radiolabeled nucleotide

		time consuming to simply ignore these template preps
	(ii) Primer site deletion	Any deletion around the primer site usually includes the insert, and the template is best ignored
(c) No sequence in specific nucleotide track	(i) Incorrect NTP mix	Most probably no dNTP in mix, or possibly extremely high ddNTP concentration
	(ii) Specific NTP mix omitted	—
(d) No sequence in single track	Reagent omitted	—
(e) Very faint sequence in set of four tracks	(i) Low template concentration	Check concentration on an HGT agarose minigel. Often, very large insert clones need a longer growth time
	(ii) Template contains very few T residues	Very rare. Use an alternative α-radiolabeled nucleotide

(f) Very faint sequence throughout	(i) Primer concentration too low	Try a titration of concentration
	(ii) Old radiolabeled nucleotide	—
	(iii) Saran wrap not removed from dry gel	—
(g) Dark background to sequences	(i) Templates of poor quality	Increase volume with TE and rephenol and re-ethanol precipitate
	(ii) Contamination with chromosomal DNA, caused by overgrowth, carryover of cells, or lysis of unhealthy cells	If the template is important, retransfect using 1 μl of 1/50 dilution of template and regrow. Otherwise discard
	(iii) Templates were picked from an old plate	As above
(h) Double sequence: each position of the ladder is represented by two bands	(i) Two templates present; one is normally a deletion product	If the template is important, retransfect using 1 μl of 1/50 dilution of template and regrow, otherwise discard
	(ii) Double priming: concentration of primer far too high or insert has sequence homologous to the priming site	Adjust primer concentration or try one of the alternative primers
(i) Sequence track fades out toward the top of the gel	Dideoxy/deoxy ratio too high	—
(j) Sequence track very faint at the bottom of the gel	Dideoxy/deoxy ratio too low	—
(k) Bands across all four tracks	Insufficient or poor-quality enzyme	Try 2 U Klenow per clone or new batch if this is no better
(l) High incidence of artifact bands	Insufficient or poor-quality enzyme	As above
(m) Bands smeared out, especially near top of gel	Associated with decomposition of urea, particularly in old gel mix	Use only deionized or ultrapure urea (make fresh gel mix after 1 month)
(n) Black speckles on autoradiograph at the top of the gel	Particles in the gel	Filter gel mixes. Let gel mix warm to room temperature before pouring to ensure urea is fully dissolved
(o) High proportion of counts retained in wells	(i) Wells dried out before loading	—
	(ii) Samples renatured before loading.	Load within 20 min of denaturation

gel (8 or 10%), the compression is close to the priming site (<50), and unmodified control sequence reactions are run alongside, it is normally possible to determine the sequence across even the most complex compressions.

Data Compilation

The accumulation of data by the random method is so rapid that a computer is a necessity for the efficient overlapping and ordering of the sequence data. A wide range of computer programs is available to run on different computers.[14] We use the DBUTIL series of programs by Staden[15] run on a VAX computer. A device for entering sequence data into the computer directly from the film is also advisable; we currently use a sonic digitizer fixed to a lightbox, and the computer program GELIN by Staden.[15] Positional information is fed into the computer by touching each band with a pen device. The sound emitted by the pen is recorded by two microphones on the digitizer, enabling the band position to be accurately calculated. The position of each band along the x axis determines which base is recorded. The program generates separate files of the sequence obtained from each clone, and another file which contains only the names of these files (the file of file names). These files can then be used by the programs SCREENV and SCREENR, which automatically check each sequence for any vector sequence or restriction sites generated by the ligation of the ends of the fragment in the self-ligation reaction. Sequences that pass this screening procedure are then entered into a database using the program DBAUTO. Each sequence is checked against any previous sequences entered into the database. Matching sequences are compared and padded when necessary to produce an optimum alignment. Sets of matching sequences, or contigs, are built up during the project, until a completely overlapping contiguous sequence is obtained. Padding characters introduced into the sequence database by the program or other mismatches can be corrected manually using the DBUTIL program, after checking any conflict of data on the original autoradiographs.

Careful examination of the database using this program is then needed to reveal any problem areas where special procedures need to be employed to resolve compressions, or to complete sequences that have only been obtained on one strand. Because compressions can sometimes be extremely difficult to spot, it is absolutely essential to obtain the sequence

[14] *Nucleic Acids Res.* 14(1) (1986) (issue devoted to application of computers to research on nucleic acids).
[15] R. Staden, *Nucleic Acids Res.* 14, 217 (1986).

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from both strands, employing the clone-turnaround method if necessary. The redundancy of sequence data generated by the random method will be useful in resolving difficult regions and gives greater confidence in the correctness of the deduced sequence.

[8] The Use of DNase I, Buffer Gradient Gel, and ^{35}S Label for DNA Sequencing

By GUO-FAN HONG

The use of microcentrifuge tubes and mixing of sequencing reactions by brief centrifugation in racks rather than the original capillary tube method has made sequencing reactions relatively simple.[1] The rate-limiting step in DNA sequencing is therefore the number of gels that can be run.

Buffer gradient gels and ^{35}S label are simple means of increasing the rate of sequence analysis; they add little time to that required for determining the sequences of a given number of clones, need no elaborate equipment, and increase the amount of useful data per gel. The standard approach of running 2- and 4-hr gels generates about 300 bases of sequence. The above improvements allow the same number of bases to be read with more confidence from a single 50-cm gel for each clone sequenced due to the changed spacing between sharpened bands. It is now possible to determine the sequences of 28 clones per day by running samples on one gel only, i.e., thousands of bases of sequence data can now be generated in a day. The sequencing reaction procedures as a whole are not the rate-limiting factor in obtaining gel readings. The cloning itself and generating the required subclones take more time.

Two major reliable DNA sequencing procedures,[2,3] which allow the sequence determination of a stretch of DNA of up to about 300 nucleotides, are available. Longer sequences must be determined in short stretches, which are then joined together on the basis of their overlaps using computer programs.

The method of generating sequential deletion subclones with DNaseI

[1] M. D. Biggin, T. J. Gibson, and G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* 80, 3963 (1983).
[2] A. M. Maxam and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* 74, 560 (1977).
[3] F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463 (1977).